

Distributional Consequences of Adhesive Eggs and Anural Development in the Ascidian *Molgula pacifica* (Huntsman, 1912)

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Abstract. *Molgula pacifica* (Huntsman) is a recently rediscovered ascidian that occupies shallow subtidal rocks on wave-swept coasts of British Columbia. Individuals occur most abundantly at sites with intermediate exposure at or near 4 m depth. On a scale of centimeters, they are highly aggregated. *Molgula pacifica* is hermaphroditic, self-fertile, and oviparous. Embryos develop on the bottom without passing through a typical tadpole stage. Each of the egg follicle cells contains a single large adhesive vacuole that occupies most of the cell volume. Shortly after spawning these vacuoles rupture, causing the follicle cells to secrete a sticky mucus coat that adheres the egg to the substratum. Juveniles hatch and move away from the chorion using epidermal ampullae, as reported for other anural molgulids. Adhesive eggs may be an adaptation that permits anural development in high-energy hard-bottom habitats. Egg adhesion may also explain the small-scale distribution of the species.

Introduction

“. . . it is very probable that (anural development) is to be correlated with the outstanding peculiarity of the family, namely, its unattached sand-flat habitat and its adaptation to such an existence.” (Berrill, 1931)

Most ascidians pass through a “urodele” or tadpole larval stage. Suppression of the tailed tadpole (termed “anural development”) is known only in the families

Molgulidae (Lacaze-Duthiers, 1874; Damas, 1902; Berrill, 1931) and Styelidae (Millar, 1954, 1962), though within the Subphylum Urochordata, tailed larvae are also lacking in the development of most thaliaceans (Berrill, 1950). Anural development has been previously observed in 10 molgulid species (Berrill, 1931), eight of which live on sandy or muddy bottoms. Only two urodele species, *Molgula oculata* and *M. occidentalis*, inhabit soft bottoms. Conversely, on hard substratum, 13 of the 15 molgulid species with known developmental modes demonstrate normal urodele development (Berrill, 1931; Whittaker, 1979; Torrence and Cloney, 1981). Both known anural styelids, *Peleonia corrugata* (Millar, 1954) and *Polycarpa tinctor* (Millar, 1962), live on sandy substrata. Berrill (1931) reasoned that urodele development is an ancestral condition that gave rise to anural development among sand-dwelling species because larval swimming and habitat selection have little value where the substratum is flat and homogeneous. By extension of this argument, he suggested that the few attached anural species represent reinvasions by sand-dwelling forms of the ancestral hard-bottom habitat (Berrill, 1931). Whittaker's (1979) discovery of vestigial tail muscle acetylcholinesterase in anural species provides indirect support for these ideas. Although we might expect that anural species colonizing rocky substrata would somehow compensate for their inability to swim, no compensatory features have been described.

Huntsman (1912) described *Molgula pacifica* from a single specimen collected at Ucluelet, British Columbia. Besides a redescription by Van Name (1945) based on this same specimen, nothing more is known about the biology of this species. We recently discovered a large subtidal population of *Molgula pacifica* in Barkley Sound, British Columbia, approximately 35 km from the

Received 23 July 1986; accepted 28 October 1987.

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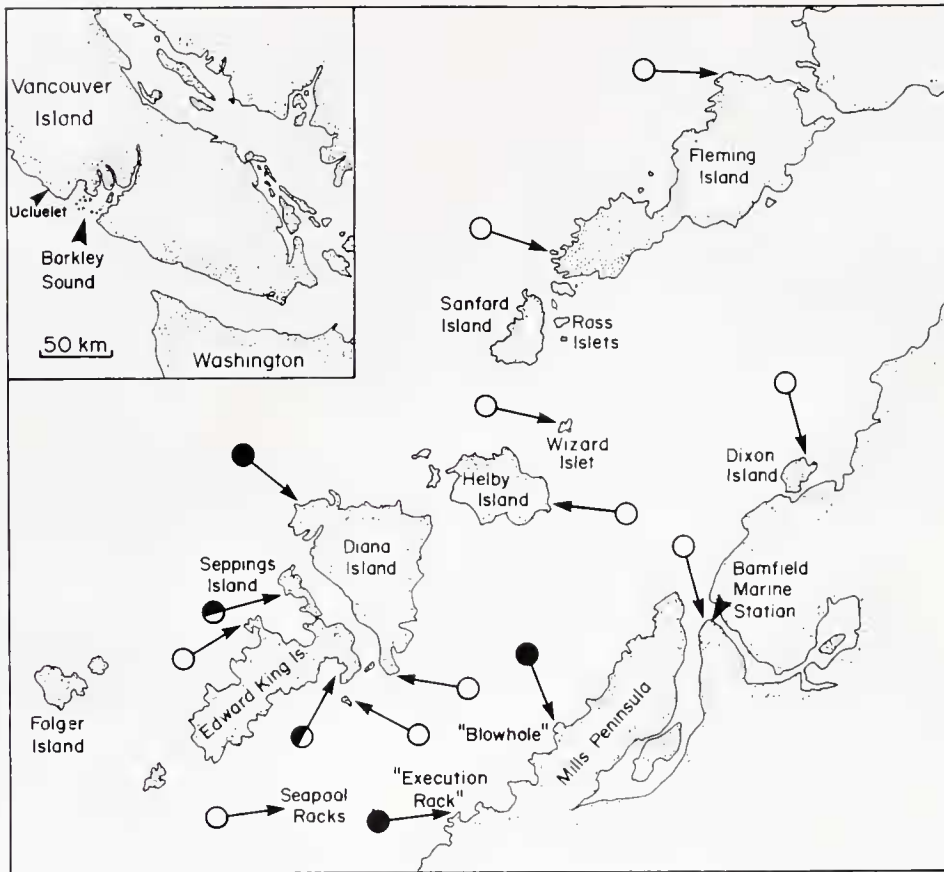


Figure 1. Map of the study region on the southeastern edge of Barkley Sound, British Columbia, Canada. Arrows indicate subtidal sites that were systematically surveyed for *Molgula pacifica* populations. Circles attached to the tails of the arrows give qualitative density values as follows: closed circle: abundant; open circle: absent; half-shaded circle: present in low numbers.

type locality. In this paper, we describe the general characteristics of this species' habitat, give a quantitative analysis of distributional patterns on several scales, present a general description of its anural development, and report an unusual developmental adaptation that may permit exploitation of high-energy subtidal habitats. A redescription of the species will be presented elsewhere (Pennechetti *et al.*, in prep.).

Materials and Methods

Distributional surveys

We studied the distribution of *Molgula pacifica* at three scales. Large-scale qualitative surveys were made at 16 sites along a 10 km exposure gradient in Barkley Sound, British Columbia. At each site, at least 4 divers began at 10 m depth and worked up the slope, stopping to search more carefully at 6 m, 4 m, and 2 m depths. No quantitative data were taken on these survey dives. Field notes consisted of observations on sizes of individuals, animal and plant associates, depth of occurrence, surface angles of occupied rocks, and wave surge.

At an intermediate scale, quantitative data were taken at the Blowhole site on the southern shore of Barkley Sound, where *Molgula pacifica* occurred abundantly. Within each of two long surge channels (called Blowhole sites 1 and 2 hereafter), we counted all individuals in five randomly positioned 50×50 cm quadrats at each of three depths (6 m, 4 m, 2 m). Within each depth, three habitat types were surveyed: (1) gentle slopes or horizontal surfaces in channel bottoms, (2) vertical surfaces on the sides of channels, and (3) horizontal or sloping ridges or plateaus between surge channels. The data for each site were analyzed by 2-way ANOVA in which both factors (depth and habitat) were fixed.

Small-scale (within-habitat) distribution was quantified from underwater photographs taken of the rock surface. At each of 4 sites (3 at Blowhole, 1 at Execution Rock), we photographed a 6×6 grid of contiguous rectangular quadrats on rock walls or steep slopes. Each quadrat encompassed an area 15×22 cm. Transparencies were projected and counted by at least three individuals before the data were compared with expected random (poisson) distributions using goodness of fit tests.

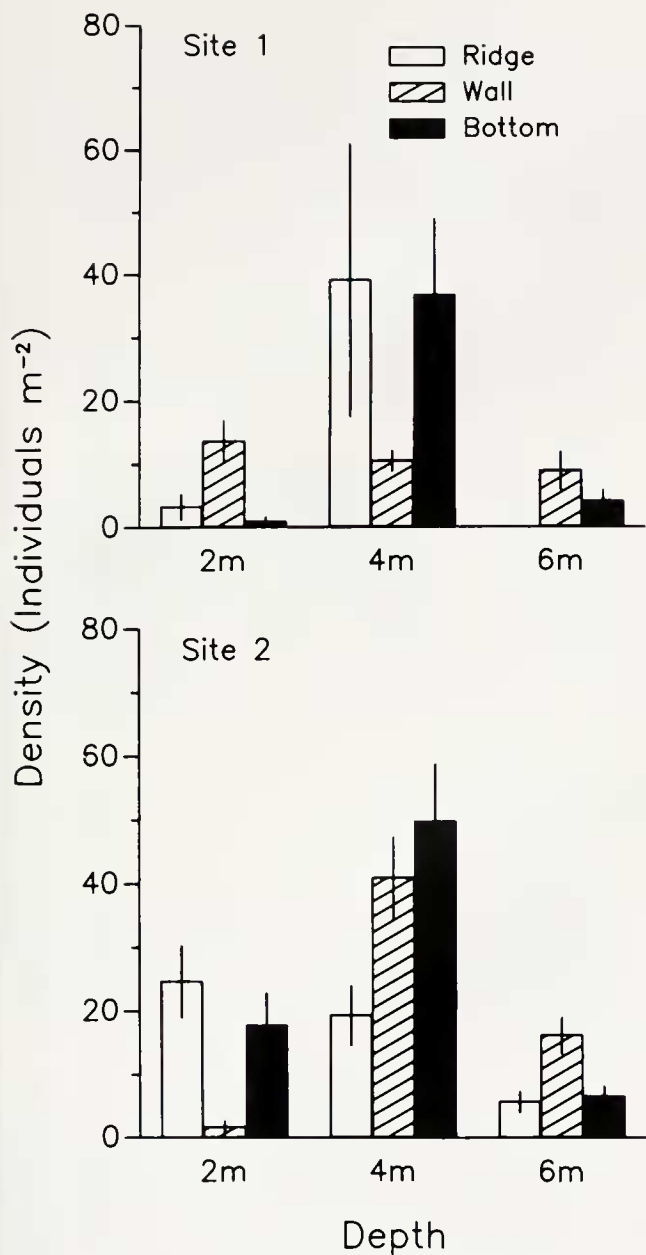


Figure 2. Densities of *Molgula pacifica* at three depth/habitat combinations in two surge channel systems at the Blowhole site. Error bars represent one standard deviation on each side of the mean. Data analysis is presented in Table 1.

Other species of solitary ascidians were also counted in one grid (Blowhole grid 1) to determine if their densities correlated with those of *Molgula pacifica*.

Embryology and histology

Individuals of *Molgula pacifica* were collected by scuba from the Blowhole site in Trevor Channel, British Columbia, during July 1985, and placed in seawater tables at the Bamfield Marine Station within one hour of collection. Gonads were removed from some adults by

dissection, then macerated through 253 μm nitex monofilament mesh. Other individuals released gametes spontaneously in seawater tables. Following fertilization, excess sperm were removed by repeated rinsing with fresh seawater. Cultures were maintained at 10°–12°C in a shallow, flow-through seawater table. We attempted to induce spawning by light shock by incubating eight individuals in darkness for 12.0 h, then exposing them to the subdued light of the laboratory.

Follicle cells of dissected eggs did not secrete adhesive spontaneously. We induced holocrine secretion for histological study by placing the eggs in hypotonic seawater (1 part seawater, 2 parts distilled water). Eggs treated in this way appeared identical to eggs spawned naturally in the laboratory.

Embryos were fixed in Torrence's fixative for 2.5 h, followed by three 20-minute rinses in Torrence's buffer (Torrence and Cloney, 1981). Embryos were fixed, rinsed, and dehydrated on ice, then brought to room temperature during the first change of absolute isopropanol. They were embedded in Luft's Epon 812 (Luft, 1961) and sectioned on a Reichert OMU2 ultramicrotome using glass knives. Thin sections were stained with uranyl acetate, post-stained with lead citrate, and examined at 60 kV using a Phillips EM 300 electron microscope. To aid in the recognition of mucus, 1- μm sections were stained with an aqueous solution of 0.25% toluidine blue in 0.5% sodium borate (Humason, 1967; R. Burke, pers. comm.).

Living embryos and juveniles were observed and photographed on Leitz or Zeiss photomicroscopes using phase contrast, bright field, or Nomarski optics.

Results

Distribution

The distribution of *Molgula pacifica* showed clear patterns at three scales. Figure 1 shows the large-scale distribution, as determined by qualitative surveys, within the southern portion of Barkley Sound and the Deer Island group. The mouth of the sound experiences the rough surf and surge conditions of the open Pacific coast. Wave action decreases regularly along the southern shore of the sound because of protection from the Deer Islands and Broken Islands. The conditions at any given site in the Deer group depend on the distance of the site from the mouth of the sound, protection by other islands, and direction of exposure. Individuals of *M. pacifica* were not found at either the most exposed sites or the most protected sites. The sites at which they were most abundant had moderate exposures with constant, year-round surge. This pattern is apparent along at least three exposure gradients (Fig. 1). Along the southeastern shore of Barkley Sound abundances went from zero at Seapool rocks to high density at Execution Rock and Blowhole,

Table I

Analyses of variance testing the importance of habitat (channel wall, channel bottom, ridge between channels) and depth (2,4,6 m) on densities of *Molgula pacifica* in two channel systems at the Blowhole site (Fig. 2)

Source of variation	d.f.	SS	MS	F	P
Site 1:					
Depth	2	2.763	1.381	23.55	0.000
Habitat	2	0.260	0.130	2.22	0.123
Depth × habitat	4	1.485	0.371	6.33	0.001
Error	36	2.112	0.058	—	—
Site 2:					
Depth	2	2.048	1.024	29.03	0.000
Habitat	2	0.092	0.046	1.30	0.284
Depth × habitat	4	2.089	0.522	14.81	0.000
Error	36	1.270	0.035	—	—

then dropped to zero at Bamfield Inlet and Dixon Island. On the leeward side of the Deer group, where the exposure gradient is more abrupt, intermediate numbers of animals were found at Edward King Island, but none were found on the southern tip of Diana Island or on the Eastern Shore of Helby Island. Finally, on the windward side of the Deer group, no animals were found on an exposed northern tip of Edward King Island, intermediate numbers were present on Seppings Island, high numbers were found on Diana Island, and abundances dropped to zero on the northwestern sides of Wizard Islet and Fleming Island.

The two parallel surge channels surveyed at the Blowhole site (Fig. 2) had different overall densities of *Molgula pacifica*, so the distributions were analyzed with separate two-way analyses of variance (Table I). The same general pattern was apparent at both sites. The main factor of depth and the depth × habitat interaction (where habitat refers to channel bottoms, sides, or the ridges between channels) were significant in both cases. At site 1, about 6 times as many *M. pacifica* occurred at 4 m depth than at either 2 m or 6 m. Qualitatively, the same pattern was seen at site 2. The interaction at site 1 resulted from most individuals occurring on the channel walls at 2 m and 6 m, but more occurring on channel bottoms and ridges at 4 m (Fig. 2). The distributions are similar between sites at 6 m, but differed substantially at 2 m and 4 m. At site 2, very few individuals occurred on channel walls at 2 m. At 4 m, approximately equal numbers occurred on the walls and channel bottoms (Fig. 2).

Frequency distributions of the abundance data from the small-scale photographic grids were compared with poisson distributions to determine the nature and significance of small-scale clumping (Fig. 3). With the exception of Blowhole grid 1, which had a much lower overall density than the other three, all of the observed

distributions differed significantly from expected random distributions. Numerous quadrats with zero values and quadrats on the upper tails of the observed frequency distributions (densities as high as 727.2 animals per m² in individual quadrats) indicate aggregated spatial distributions. To visualize the nature of the clumps, we depict the raw data as three-dimensional plots (Fig. 4). Each grid encompassed a rectangular area 1.188 m². Clumps were generally on the scale of several quadrats (*i.e.*, tens of centimeters). At Execution Rock, on a gently sloping rock face, there was a sharp density gradient that went from a region with few animals in one corner to a single large aggregation that occupied most of the remainder of the plot.

To determine if the aggregations of *Molgula pacifica* occupied the same sites as aggregations of ascidians with tailed tadpoles, we computed correlation coefficients in which *M. pacifica* abundances were paired with abundances of three other species occurring at Blowhole grid 1. Two of the species, *Pyura haustor* and *Chelyosoma productum*, are strongly gregarious as larvae, whereas the third, *Cnemidocarpa finmarkiensis*, settles randomly

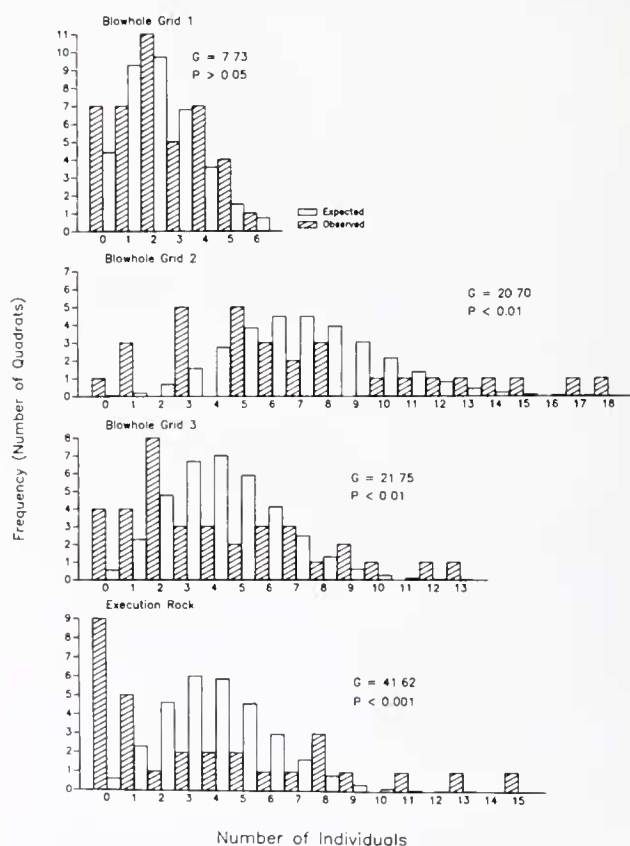


Figure 3. Observed (shaded bars) and poisson (open bars) frequency distributions of *Molgula pacifica* in small-scale photographic grids. In computing the goodness of fit (G) values for each site, classes with expected frequencies less than 2.5 were combined on the tails to make the significance tests more conservative.

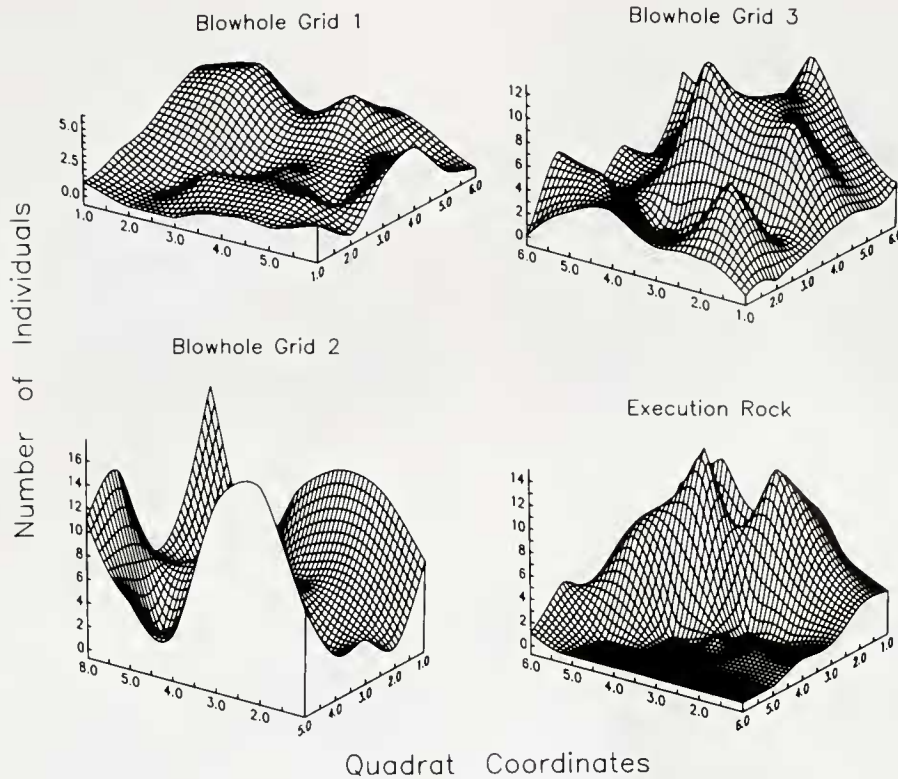


Figure 4. Small-scale abundance patterns of *Molgula pacifica* at four sites.

with respect to established individuals (Young, 1982; Young and Braithwaite, 1980). None of the correlation coefficients were significant (*P. haustor*: $r = 0.005$; *C. productum*: $r = -0.002$; *C. finmarkiensis*: $r = -0.244$; $P > 0.05$ for all three).

Reproduction and development

Unlike many ascidians that spawn during the day following dark adaptation, *Molgula pacifica* released gametes during periods of darkness. All eight individuals tested spawned, and all released gametes during, not after, the dark adaptation period. Approximately 100–200 eggs were released by each individual. Adults were maintained in separate dishes during these experiments, yet all embryos were undergoing development when they were discovered in the morning. In dissecting numerous specimens we never found embryos being brooded within the atrial chamber. Thus, *M. pacifica* is an oviparous hermaphrodite capable of self fertilization.

The eggs are 180 μm in diameter, including the follicle cells. At spawning, the egg has a single outer layer of follicle cells surrounding the chorion (Fig. 5). The chorion is closely applied to the egg plasma membrane; thus, the perivitelline space is much smaller than in most typical oviparous ascidians (Fig. 6). Sections of the eggs revealed no test cells (Fig. 5, 6), but careful observation of living

material showed a few test cells present (R. Cloney, pers. comm.). Each follicle cell contains a single large vacuole which occupies most of the cell volume. Shortly after spawning, these vacuoles undergo a holocrine secretion of their contents to form the adhesive coat (Fig. 7, 8). This coat, which stains intensely with toluidine blue, swells to occupy a space many times the volume of the original follicle cell (Fig. 9). Those portions of the follicle cell membranes not disrupted by the secretory process remain closely applied to the chorion after secretion (Fig. 8), but all traces of the follicle cells disappear by 24 h after fertilization.

All but a few of the naturally spawned eggs were firmly attached to the substratum at the time they were discovered. Many of these were clumped together and joined by a common adhesive coat (Fig. 9).

Eggs removed from the ovaries by dissection did not produce adhesive coats spontaneously. Nevertheless, many were sticky and their follicular vacuoles could be induced to release their contents by exposure to a hypotonic medium. Following secretion, such eggs appeared identical to naturally spawned eggs with adhesive coats.

Complete embryonic development occurs within the adhesive coat; no tailed tadpole larva is ever produced. The early details of anural development in this species are obscured by the opacity of the adhesive coat and embryo. At 10–12°C, the first juveniles hatched 36 h after

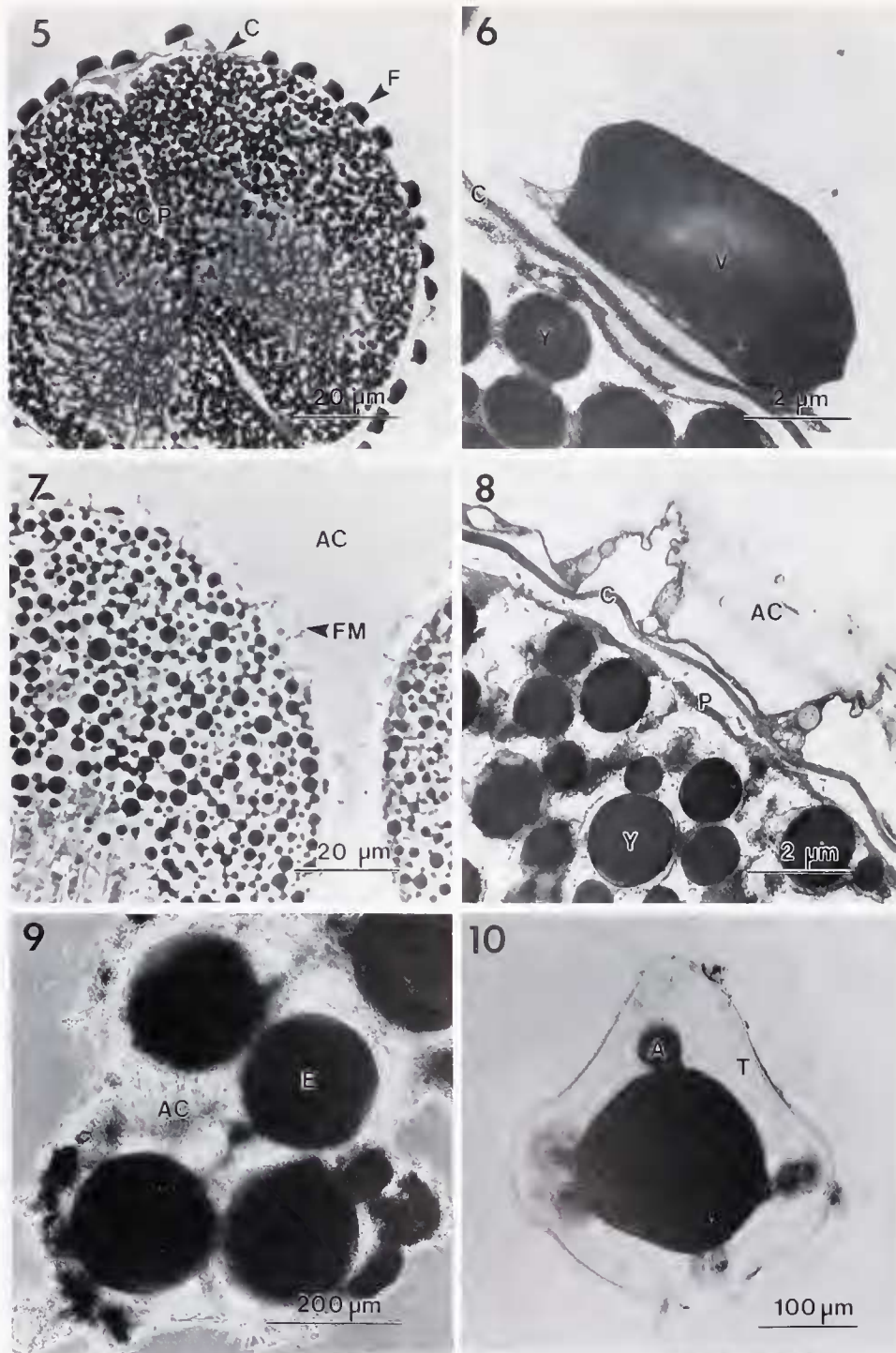


Figure 5. One-micron section of naturally spawned 2-cell embryo just prior to secretion of the adhesive coat. Note the very thin perivitelline space and apparent absence of test cells. Stained with toluidine blue. F: follicle cell. C.P.: cleavage plane. C: chorion.

Figure 6. Electron micrograph of a follicle cell prior to secretion of adhesive coat. V: vacuole. C: chorion. Y: yolk granule.

Figure 7. Thick (1- μ m) section of an egg following secretion of the adhesive coat. AC: adhesive coat. FM: follicle cell membranes.

Figure 8. Electron micrograph of an artificially removed egg just after secretion of adhesive has been induced by exposure to hypotonic seawater. C: chorion. P: plasmalemma. AC: Adhesive coat. Y: yolk granule.

Figure 9. Aggregation of naturally spawned eggs (E) bound together by common adhesive coat (AC).

Figure 10. Juvenile shortly after hatching. A: ampulla. T: tunic.

fertilization. Just before hatching, a variable number (1–5) of epidermal ampullae were extended onto the substratum (Fig. 10). As in other molgulids, embryos hatch by rupture of the chorion. One ampulla was often larger than the others, and juveniles generally extended this ampulla through the initial fissure in the chorion. No embryos were observed to hatch before any ampullae had formed, but some hatched with only a single ampulla. The broken adhesive coats remained attached to the substratum for several days after hatching, then deteriorated.

Discussion

Molgula pacifica is one of only three molgulids known to have anural development while occupying hard substratum, and it is the only known anural species in the Pacific Ocean. It is particularly surprising that the species lives in wave-swept habitats. We propose that the adhesive eggs may be an adaptation that permits this unexpected habitat distribution, as the eggs probably stick to surfaces near the parents and develop on the bottom. Tadpoles of other species living in this habitat presumably use their swimming ability to assist in reaching the substratum.

The highly clumped small-scale distribution may be explained by two mechanisms associated with egg adhesion. First, eggs frequently attach to each other after spawning; indeed, they often share a common adhesive coat. Such egg masses falling to the bottom would place numerous individuals in close proximity. Although ampullar locomotion probably disperses individuals away from the initial attachment site at hatching, such locomotion would not be expected to obliterate the clumped pattern. Aggregations produced in this manner by siblings should be dominated by single size classes. Second, local aggregations could be established and maintained by limited (philopatric) dispersal. It seems reasonable to assume that eggs should be more likely to contact the substratum near their parents than further away. These juveniles in turn would produce short-distance dispersing offspring of their own, resulting in aggregations with polymodal size distributions. We were unable to make precise size measurements of individuals in our photographs because of adjacent epifauna. Nevertheless, it is clear from the photos that clumps tend to contain animals of all sizes, suggesting that the second method of aggregation could be important.

Philopatric dispersal is known in many other sessile organisms (reviewed by Jackson, 1986), including some that occupy high-energy habitats. For example, the intertidal alga *Postelsia palmaeformis*, which lives only on exposed headlands, often forms aggregations containing individuals of all sizes (Dayton, 1973). During low tides, spores are released from sori located in grooves of the

drooping blades. The spores flow down the grooves and fall on or near the haptera of the parents, where they adhere (Dayton, 1973).

Some ascidians with active tadpole larvae settle gregariously, whereas others settle randomly (Young, 1982; Young and Braithwaite, 1980). Both kinds of species occurred in the same habitats as *Molgula pacifica*. *Chelyosoma productum*, which aggregates behaviorally *in vitro* (Young and Braithwaite, 1980) and in the field (Young, 1982) formed clumps at about the same scale as those of *M. pacifica* at our study sites. However, the densities of the two species were not correlated, suggesting that the aggregations are formed and maintained independently. *M. pacifica* densities were also not correlated with those of *Pyura haustor*, another gregarious species, nor with *Cnemidocarpa finmarkiensis*, a species that does not aggregate. If some sites were consistently better for juvenile survival than others, or if current patterns concentrated propagules in certain regions, we would expect positive correlations among ascidian species since neither of these processes should discriminate among tadpoles of different species. The low correlation coefficients do not support the idea that ascidians accumulate passively. Thus, clumps of *Chelyosoma productum* and *Pyura haustor* are probably established by tadpole behavior, whereas similar clumps of *M. pacifica* are probably formed by philopatric dispersal mediated by sticky eggs.

In the egg of a typical urodele ascidian, test cells are located within superficial concavities of the oocyte (Kessel and Kemp, 1962). A single layer of inner follicle cells is separated from the oocyte and test cells by the chorion. Prior to ovulation, a layer of outer follicle cells surrounds the inner follicle cell layer (Kessel, 1983). At ovulation, the outer follicle cell layer remains in the ovary with the germinal epithelium, the chorion lifts away from the plasma membrane of the oocyte, and the test cells are extruded from their superficial concavities and come to lie within the perivitelline space. The eggs of some anural ascidians (e.g., *Molgula bleizi*) contain test cells and a perivitelline space, whereas the eggs of others (e.g., *M. retortiformis*) have neither (Berrill, 1931). The eggs of *M. pacifica* have very few test cells lying within a perivitelline space much smaller than that seen in typical urodele species.

The functions of the follicle cell layer vary among eggs from different ascidian species. The follicle cells of *Corella inflata* are filled with ammonia and cause the eggs to float (Lambert and Lambert, 1978). Highly vacuolated follicle cells of other species probably reduce the egg's specific gravity to slow the sinking rate of the egg (Harvey, 1927; Berrill, 1931). Kessel and Kemp (1962) described a secretory product in the follicle cells of *Ciona intestinalis* and *Molgula manhattensis*. However, these secretory masses break down during oocyte maturation and are not present at ovulation; at spawning, the eggs

contain a granular material of unknown function. *M. pacifica* is the only species reported to have secretory follicle cells after ovulation and the only one whose follicle cells function in attachment. The cells themselves are essentially destroyed when the adhesive material is created.

Lucas (1927) reported that the eggs of *Molgula robusta* are often held together by strings of adhesive mucus which facilitate dispersal by reducing sinking rates. These strings could be wafted from the bottom of the culture dishes by stirring, a feat that would be impossible with the securely attached eggs of *M. pacifica*.

Kupffer (1875; cited in Berrill, 1931) argued that anural development was the forerunner of urodele development, and Lacaze-Duthiers (1877) classified anural and urodele species in separate genera. However, virtually all later authors consider direct development to be the derived state (Berrill, 1931). Anural development is probably polyphyletic (Berrill, 1931). It has arisen independently in at least two different families (Styelidae and Molgulidae), and within the Molgulidae it has probably arisen in at least four different clades (Berrill, 1931; Huntsman, 1922). Berrill (1931) argues convincingly that in each case anural development arose as an adaptation to sedimentary environments, where neither swimming nor habitat selection had a strong selective advantage. It follows then that the few anural species occurring on hard bottom represent reinvasions of the original habitat by species that had evolved the soft-bottom developmental mode. In *Molgula pacifica*, adhesive eggs may be one character that facilitated such a reinvasion and that allowed the species to recruit and maintain populations on hard bottoms in surgy habitats just as well as species with typical tadpole larvae.

Acknowledgments

We thank R. E. Foreman, director of Bamfield Marine Station, and R. L. Shimek, associate director, for providing lab facilities and logistical support for the Ascidian Biology course during which this project was initiated. A. Bergey, R. Shimek, and S. Smith assisted with field work. J. L. Cameron, B. L. Bingham, R. R. Olson, and two anonymous reviewers improved the manuscript with their comments. J. L. Cameron and B. L. Bingham also assisted with data analysis and computer plotting. We are especially grateful to R. A. Cloney for his timely assistance during the course and his critical review of an earlier manuscript. Harbor Branch contribution number 613.

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